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Ovarian cancer is the fourth leading cause of cancer-related death in U.S. women. This program project approaches the ovarian cancer problem by 1) establishing a human ovarian tissue and clinical data base core facility to support the proposed projects and future investigations, 2) identifying genes which are differentially expressed in ovarian cancers and thereby discovering biomarkers for early detection, 3) studying ovarian tumorigenesis in ovarian tissues obtained from germline BRCA1 mutation carrier to better understand the interaction between mutational inactivation of BRCA1, the cellular caretaker gene and p53, the cellular gatekeeper gene, and 4) developing a genetically defined mouse model of epithelial ovarian cancer.

To date, the ovarian tissue core has banked over 260 surgical specimens and provided sufficient resources for the ongoing projects and other collaborative research on ovarian cancer etiology. Representational difference analysis was used to identify 160 gene specific for normal ovarian epithelium and 95 genes specific for ovarian cancer. The BRCA1-mutation associated ovarian tissues required to understand the functional interaction between of p53 and BRCA1 have been identified. And, a new viral construct carrying the cre recombinase under the control of the K18 promoter has been tested to establish its ability to mediate recombination in mouse ovarian epithelial cells.

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INTRODUCTION:

It is estimated that 25,400 women will be diagnosed with ovarian cancer in the year 2003, and 14,300 women will succumb to this disease. In recent years, we have seen a slight decline in the number of new cases of ovarian cancer, and survival from ovarian cancer has been prolonged by improvements in surgery and chemotherapy. However, substantial progress towards ultimately eliminating ovarian cancer as a threat to women has been undermined by our ignorance about its etiology. Without additional insight into the genetic alterations that result in the clinical entity of ovarian carcinoma, we are left with empiric approaches to prevention, early detection and therapy. This program project is aimed at approaching the ovarian cancer problem by 1) establishing a human ovarian tissue and clinical data base core facility to allow the successful completion of the proposed projects and future studies aimed at understanding and eradicating ovarian cancer, 2) identifying genes which are differentially expressed in ovarian cancers and using this information to discover biomarkers for early detection, 3) studying ovarian tumorigenesis in "high risk" ovarian tissues obtained from carriers of germline *BRCA1* mutations to better understand the interaction between mutational inactivation of *BRCA1*, the cellular caretaker gene and *p53*, the cellular gatekeeper gene, and 4) developing a genetically defined mouse model of epithelial ovarian cancer which would be a vital tool for further studies of ovarian cancer etiology, prevention and therapy. This addendum to our final report prepared for the USAMRMC updates our progress towards achieving these goals.

BODY:

Ovarian Tissue and Clinical Database Core Facility
Beth Y. Karlan, MD, Principal Investigator

The late stage of clinical presentation characteristic of human ovarian carcinoma is responsible for the tragically high mortality rates associated with the disease. To address this problem, we drew on the rich human ovarian tissue resources available at Cedars-Sinai Medical Center and UCLA. The Ovarian Tissue and Clinical Database Core Facility has been the cornerstone of this program project designed to define the genetic alterations and phenotypic determinants of human ovarian carcinoma. Patient accrual to the Ovarian Tissue Bank Core was completed at the end of Year 3 in September 2002. The Tissue core continues to provide a rich resource of clinical specimen from patients with ovarian and peritoneal adenocarcinoma and from patients at high risk of ovarian cancer due to a genetic predisposition. The *in vitro* models established by the core continue to be maintained and utilized to facilitate research aimed at understanding genetic mechanisms involved in ovarian carcinogenesis and preclinical investigations of molecular-based therapeutics. The core facility has also built resources to support future studies on ovarian cancer etiology, prevention and treatment.

One of the important strengths of the Core facility is its state-of-the-art relational database system that links all patient demographic, epidemiologic, medical and clinical information with each banked specimen and resource. This link facilitates translational research that will allow us to make clinical correlations with basic research findings and makes it possible to transfer basic laboratory findings to the clinical arena. In addition, the database was designed to facilitate a multidisciplinary approach to ovarian cancer research and therefore includes additional fields for data entry that will support future studies including behavioral, and environmental influences and prevention studies. The database continues to be maintained with the additional follow-up clinical data and utilized for translational research studies.

The Tasks as outlined in the protocol were completed at the end of year 3, September 30, 2002. The accomplishments achieved during that period and as they have been continued are outlined here.

TASK 1: Provide a continuing resource of normal and carcinomatous ovarian tissues from patients with benign gynecologic conditions and sporadic and familial ovarian cancer, respectively (months 1-36):

Provide the requisite ovarian tissues for the proposed projects (months 1-36). The core provided all ovarian tissues requested by project principal investigators for the proposed projects. These included: 16 snap frozen tumor specimen to Project 1 (D. Chang, PI) of

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variable histologies (12 papillary serous, 1 clear cell, 3 endometrioid) and 5 snap frozen normal ovarian tissue specimen.

Continue tissue, DNA and serum procurement procedures according to established protocols at Cedars-Sinai Medical Center (months 1-36).

The tissues banked and primary cultures established are as follows:

Surgical Specimen (Ovary/PPC only):

	Total	Cancer	Benign
Y1	103	58	45
Y2	64	38	26
Y3	95	56	39
Total	262	152	110

Primary Cultures [attempts/frozen (percent)]

	CSOC	HOSE	HOST
Y1	12/28 (43%)	9/18 (50%)	9/17 (53%)
Y2	9/17 (53%)	10/12 (83%)	8/11 (73%)
Y3	9/10 (90%)	5/5 (100%)	5/5 (100%)
Total	30/55 (55%)	24/35 (69%)	22/33 (67%)

Establish protocols for specimen acquisition at neighboring medical centers' services by collaborating gynecologic oncologists (months 1-6).

Expand the Core Facility's ovarian tissue resource by collecting specimens from neighboring medical centers participating in these activities (months 7-36).

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Obtain *BRCA*-linked ovarian tissues from collaborators in Toronto and Tel Aviv to enrich the Core Facility's collection of these valuable tissues (months 1-36)

Very strict Institutional Review Boards regulations for protecting the rights of human subjects involved in research continue to impede the banking of otherwise discarded surgical specimen from outside institutions. Never the less, we obtained IRB approval from UCLA and Olive View Medical Centers for the banking of surgical specimen and blood samples and we have accrued a limited number of patients from these institutions. All contacts and procedures remain in place and we continue patient accrual from these external sites.

Determine *BRCA* mutation status on consenting ovarian cancer patients and screening program participants. Store and highlight these DNA and ovarian samples (when available) (months 1-36).

Genomic DNA samples have been isolated from blood specimen from all patients with a family history of ovarian and/or breast cancers. These specimens were sent to our collaborators in Canada for *BRCA* mutation screening. Eleven additional *BRCA1* and 2 mutation carriers have been identified from banked tumor tissue. Mutation status of these patients has been recorded in the Core's Clinical Database.

Disseminate announcement of the Core Facility's services to the scientific community in Southern California and provide ovarian tissues to approved projects (months 24-46)

The availability of services and resources from the Ovarian Tissue and Clinical Database Core Facility was disseminated to investigators nationally and in Southern California via oral communication and through recognition of the Core Facility and DOD funding at scientific meetings and on research publications. After reviewing requests, we provided resources to the two following investigators: Michel Schummer, Institute for Systems Biology, Seattle Washington received 100 µg RNA isolated from 5 HOSE cultures to use for microarray analysis; Kurt Gish, Ph.D., EOS Biotechnology Inc., South San Francisco, in collaboration with Rae Lynn Baldwin, Ph.D. and Beth Karlan, M.D (Core Co-Investigator and PI, respectively) received snap frozen tissue specimen from 44 ovarian adenocarcinoma and 10 benign ovaries for use in microarray analysis and one ascites specimen for monoclonal antibody screening.

TASK 2: Establish, characterize and maintain monolayer cultures of human ovarian epithelial cells, human ovarian stromal cells and human ovarian carcinomas according to established procedures (months 1-36)

Provide the necessary primary ovarian epithelial cell cultures for the proposed projects (months 1-36)

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The project investigators did not request ovarian epithelial cell cultures during this funding period.

Cryopreserve characterized primary ovarian epithelial cell cultures (months 1-36)

Continue to establish, characterize and maintain additional primary human ovarian cultures for ongoing and future laboratory experiments, since these cells senesce after approximately three months of growth *in vitro* (months 1-36)

See data summary under Task 2.

TASK 3: Expand and maintain the clinical database to serve as an ongoing resource for translational studies (months 1-36)

Enter demographic, epidemiologic and medical history data on patients contributing tissue specimens to the Core Facility (months 1-36)

Obtain the pathology and operative reports correlating with the stored tissues and inter data into the computerized system (months 1-36)

Obtain clinical outcomes data from patient charts and tumor registry for database entry (months 1-36)

Maintain an inventory of all stored human specimens, including tissue, serum, ascites, urine and DNA, with special care to protect patient confidentiality (months 1-36)

Enter laboratory data on monolayer cultures developed from banked specimens, such as their immunohistochemical characteristics, number of vials stored, assay number, etc. (months 1-36)

Clinical, epidemiologic and demographic data as well as specimen inventory information was entered as samples were collected and banked. We are actively formatting historical data on previously banked specimen for downloading and enhancing our reporting capabilities. The major accomplishments for of downloading historical data during this funding period include:

1. Inventory and growth records of all primary cultures
2. Characterization IHC data from all cultures
3. Inventory of viably frozen primary cell cultures
4. All culture data is linked to patient and patient cancer histopathology data

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The compilation of this historical data into the Oracle-based database has been a major undertaking that is all ready giving productive results for research design and data acquisition and analysis.

We also continue to make minor enhancements to improve and expand the utility of the database. Some of the enhancements made to the Database during this funding period include:

1. Created new screen to store records and inventory of archival pathology slides.
2. Added a new column to record date that patient informed consent was signed that is linked to the IRB number.
3. Created a new report to tract patient who sign informed consent but for which surgical specimen were not collected due to unavailability. We continue to use the web-based version of BrioQuery Explorer for free form data query design and formatting.

Project #1, Molecular Biomarkers in Ovarian Cancer
David D. Chang, MD, PhD Principal Investigator

Cancer diagnosis is based on the detection of features that are unique to transformed cells. Each unique phenotype displayed in cancer cells must be accompanied by changes in gene expression. The genes that are differentially expressed in ovarian cancers compared to their normal counterparts therefore constitute logical candidates for molecular biomarkers for cancer detection. In Project 1, we proposed to conduct a detailed analysis of gene expression differences underlying human ovarian carcinogenesis and use the information to develop biomarkers for ovarian cancer.

TASK 1: To clone genes that are differentially expressed in ovarian cancer and determine their expression profile (months 1-12).

The main objective of our project is to study the gene expression differences underlying human ovarian carcinogenesis. As proposed in Task 1 of the original application, we have conducted a representational difference analysis (RDA) using primary cultures of normal human ovarian surface epithelium (HOSE) and Cedars-Sinai ovarian carcinoma (CSOC). The rational for using cultured ovarian epithelial cells for gene expression analysis was based on the fact that the epithelial cells, which give rise to ~90% of ovarian cancer, constitute a very small fraction (<1%) of the total ovarian mass. We hypothesized that using primary cultures of normal and malignant ovarian epithelium for differential gene expression analysis would preferentially identify epithelial cell-specific genes. We have successfully identified 160 HOSE-specific and 95 CSOC-specific genes from our initial analysis, which employed HOSE and CSOC cultures obtained from two different patients. The expression of these cloned genes were surveyed in 5 additional HOSE and 10

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additional CSOC cultures to identify 46 HOSE-specific and 14 CSOC-specific genes that exhibited at least 2.5-fold difference in expression level. Based on this encouraging result, we expanded our differential gene expression analysis into a gene expression profiling study. Using oligonucleotide-based microarray chips capable of assessing expression of >12,000 genes, we examined the gene expression patterns in 10 HOSE and 22 CSOC samples.

The gene expression profiling studies revealed several promising targets that could be further pursued in biomarker studies. Included in the list of genes we are further characterizing are periostin (PN), transglutaminase-2, several protease inhibitors, and PDGF receptor. We have further investigated the expression of PN and found it to have a strong oncofetal pattern of expression and be abundantly present in ascites of ovarian cancer patients. Purified PN supported integrin dependent cell adhesion, prompting us to initiate additional studies directed at testing a hypothesis that PN is involved in peritoneal spread of ovarian cancer.

TASK 2: To characterize the protein coding information and subcellular localization of the differentially expressed genes (month 9-24)

In parallel to the above gene expression studies, we have started biochemical studies on selected genes as proposed in Task 2. In particular we have characterized a gene known as periostin (or osteoblast specific factor 2). Pertinent findings from this analysis include: (i) periostin is a secreted protein; (ii) malignant ascitic fluid contains high amount of periostin; (iii) periostin mediates $\alpha v \beta 3$ and $\alpha v \beta 5$ integrin dependent cell adhesion and migration of ovarian epithelial cells. We plan to extend this line of analysis by examining whether periostin promotes intraperitoneal spread of ovarian cancer cells in year 2.

TASK 3: To study the utility of differentially expressed genes as molecular biomarkers for ovarian cancer (months 21-36)

Using a subtractive cloning and a chip-based gene expression profiling approach, we have successfully identified target genes that could be developed into biomarkers for ovarian cancer. In addition, the newly established immortalized ovarian epithelial cell lines will be used to study the functions of the genes that are differentially expressed in cancer-derived ovarian epithelial cells.

Dr. Chang left UCLA at the end of the 2002 having made significant inroads towards achieving this project's goals. There are no new results to report at this time.

Project #2, Interactions Between *BRCA1* and *p53* Mutations in Human Epithelial Ovarian Carcinogenesis

Mark D. Pegram, MD, Principal Investigator

The molecular/genetic alterations responsible for the genesis of epithelial ovarian cancer are poorly understood. Recent molecular epidemiologic studies have defined a role for the *BRCA1* tumor suppressor gene in familial breast and ovarian cancer syndromes. Using a full-length sequencing strategy, we have recently identified a high incidence (62/108) of *p53* tumor suppressor gene mutations in sporadic epithelial ovarian cancers. This finding has recently been confirmed in our screen for research subjects in a prospective randomized trial of *p53* adenovirus gene therapy for newly diagnosed ovarian cancers in which ~70% of screened subjects had sequence confirmed *p53* mutations (which was a criteria for eligibility in this clinical trial).

Several lines of experimental evidence suggest that *BRCA1* and *p53* may act in concert in DNA damage response and repair pathways: 1) both *p53* and *BRCA1* are physically altered in response to DNA damage, *p53* by stabilization and *BRCA1* by hyperphosphorylation and nuclear relocalization, 2) *p53* and *BRCA1* molecules have a direct physical interaction, 3) both *p53* and *BRCA1* activate P21/WAF-1/cip1 as a common target gene, 4) *BRCA1* is a transcriptional co-activator of *p53*, and 5) early embryonic lethality in *BRCA1* knock-out mice is partially rescued by mutation of *p53*. Furthermore a very high percentage of breast cancers with *BRCA1* mutations exhibit *p53* mutation. We hypothesize that familial ovarian cancer tumorigenesis caused by mutational inactivation and allelic loss of the cellular caretaker gene *BRCA1* requires the mutational or functional inactivation of the cellular gatekeeper gene *p53* which controls cell cycle checkpoints and/or directs cells to undergo apoptosis. If our hypothesis is correct then we expect to find mutational or functional inactivation of *p53* in 100% of ovarian cancers from *BRCA1*-affected individuals. To test this hypothesis we have proposed collection and characterization of *p53* expression levels and mutational status in ovarian cancers from a cohort of known *BRCA1* mutation carriers. This project is relevant to understanding the etiology of familial ovarian cancer, which accounts for 10% of all ovarian cancers and, as such, represents a significant public health problem. Moreover, greater understanding of the biology of familial ovarian cancer will lead to improved diagnostic techniques which may have the potential to be exploited clinically in the management of patients with this disease. For example, a patient with known germline *BRCA* mutation and evidence of *p53* mutation in preneoplastic ovarian tissue may be at especially high risk for malignancy and prophylactic intervention either with surgery or with participation in biochemical prevention trials may be appropriate. The hypothesis that *p53* tumor suppressor function is required for *BRCA1*-linked ovarian tumorigenesis is testable using human tissue resources.

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The progress on the aims and objectives originally proposed for Project #2 are outlined as follows:

OBJECTIVE 1: To assess the frequency of *p53* gene mutations in epithelial ovarian cancers with known mutations of the *BRCA1* gene.

Ovarian carcinomas with *BRCA1* mutation are being screened for nuclear accumulation of aberrant *p53* protein using immunohistochemical staining techniques. In addition, DNA extracted from tumor material is being subjected to full-length sequencing (exons 2-11) of the *p53* transcript to identify mutations in *p53* resulting inactivation. For cases identified as having wild-type *p53*, we are seeking evidence for functional inactivation of *p53* such as that caused by *mdm-2* overexpression. In addition, we are conducting a comparison of *mdm-2* expression between *BRCA1*-mutant and wild-type *BRCA1* tumors against a background of wild-type *p53*. Such comparison should help to shed light on the role of *BRCA1* on *p53*-dependent *mdm-2* gene expression in clinical specimens *in vivo*. Finally, in order to determine whether the *p53* mutational frequency is increased as we hypothesized in *BRCA1*-mutant ovarian cancer, the incidence of *p53* mutation in *BRCA1*-linked ovarian cancer will be statistically compared to our database of 108 sporadic ovarian tumors which have previously been characterized for *p53* mutation by full-length sequence analysis of coding exons. Our efforts thus far in this project have been significantly hampered by new HIPAA regulations regarding genetic screening of archival tumor material from human subjects. Our IRB has not given us sufficient permission to execute all of the proposed work using our own banked tumor materials since sequence analysis for *p53* was not specifically called for in the original informed consent documents at the time of tissue procurement, which in some cases dates back many years. Moreover, it is now impossible to obtain informed consent from many of the individuals who have succumbed to their disease and are now not available for re-consenting for this project. According to IRB, our only recourse will be to consider procuring samples prospectively with full patient informed consent on newly identified cases. This will significantly delay data acquisition and unfortunately is beyond the scope of the timeline outlined in our original proposal.

OBJECTIVE 2: To determine if overexpression of wild-type *p53* is sufficient to induce cell cycle arrest/apoptosis in *BRCA1*-mutant ovarian cells.

Ovarian carcinoma cell lines (CSOC) with defined *BRCA1* mutation are being characterized for co-existent *p53* tumor suppressor gene mutation by full-length sequencing of *p53*. *p53/BRCA1* mutant cells are being transfected with wild-type *p53* using an adenoviral expression vector (E1a and E1b-deleted) containing a strong (CMV) eukaryotic promoter with subsequent analysis of cell proliferation, cell cycle distribution, induction of apoptosis, and induction of P21/WAF1/cip1 expression. Cellular responses to *p53* transduction in

BRCA1-mutant CSOC are then be compared directly to *p53*-mutant cells with wild-type *BRCA1*.

To determine the safety, gene transfer, host immune response, and pharmacokinetics of a replication - deficient adenovirus encoding human, recombinant, wild - type *p53* delivered into the peritoneal cavity (i.p.) alone and sequentially in combination with platinum-based chemotherapy, of patients with recurrent ovarian, primary peritoneal, or fallopian tube cancer containing aberrant or mutant *p53*, adenoviral was administered i.p. to three groups of patients with heavily pretreated recurrent disease. Group 1 (n=17) received a single dose of *p53* adenovirus escalated from 7.5×10^{10} to 7.5×10^{12} particles. Group 2 (n=9) received two or three doses of vector given alone for one cycle, and then with chemotherapy for two cycles. The vector dose was further escalated to 2.5×10^{13} particles /dose in group 2. A third group (n=15) received a 5-day regimen of *p53*-adenovirus given at 7.5×10^{13} particles /dose per day i.p. alone for cycle 1 and then with intravenous carboplatin/paclitaxel chemotherapy for cycles 2 and 3. No dose- limiting toxicity resulted from the delivery of 236/287 (82.2%) planned doses of the *p53*-containing adenoviral vector. Fever, hypotension abdominal complaints, nausea, and vomiting were the most common adverse events. Vector-specific transgene expression in tumor was documented by RT-PCR in cells from both ascitic fluid and tissue biopsies. Despite marked increases in serum adenoviral antibody titers, transgene expression was measurable in 17 of 20 samples obtained after two or even three cycles of vector administration. Vector was detectable in peritoneal fluid by 24 hours and persisted for as long as 7 days whereas none was detected in urine or stool. There was poor correlation between CT scans and CA125 responses. CA125 responses, defined as a greater than 50% decrement in serum CA125 from baseline, were documented in 8 of 16 women who completed three cycles of the multidose regimen. We note that CT scans are not a valid measure of response to i.p. *p53* adenovirus due to extensive adenoviral – induced inflammatory changes. Intraperitoneal *p53* adenovirus was safe, well tolerated, and combined with platinum-based chemotherapy can be associated with a significant reduction of serum CA125 in heavily pretreated patients with recurrent ovarian, primary peritoneal, or fallopian tube cancer.

OBJECTIVE 3: Determination of the frequency of *p53* mutations in "normal" ovarian surface epithelium from *BRCA1*-affected individuals undergoing prophylactic oophorectomy.

Evidence for *p53* mutation in ovarian surface epithelium from normal ovary is being sought by *p53* immunostaining. Identification of early *p53* mutation may help to identify subjects with an especially high risk of subsequent invasive ovarian cancers. We hypothesize that there may be increased expression of wild-type *p53* in ovarian epithelium from *BRCA1*-linked ovaries compared to *BRCA1* wild-type ovaries due to activation of *p53* by DNA damage induced by the permissive conditions of a *BRCA1* mutant background.

The progress made to date on each task according to the Statement of Work is as follows.

TASK 1: Identification and histologic analysis of ovarian cancer specimens with sequence-verified germ line *BRCA1* mutations (Months 1-36).

This task is relevant to Objective I of the proposal. During the initial year of funding we had identified ~71 malignant ovarian tumors in the Core Ovarian Tumor Bank for which peripheral blood lymphocyte DNA was available for *BRCA* gene mutational analysis. Since that time we have continued to add to this number at the anticipated rate of 10 - 15 cases per year via the ongoing Core Project effort at UCLA and Cedars Sinai Medical Centers. We have been unable to obtain more samples through our originally proposed collaborative efforts because of new IRB restrictions on identification of human subjects with known germ line molecular genetic alterations (HIPAA regulations). We are thus unfortunately not legally allowed to identify potential participants for this study based on tumor bank data registry of known *BRCA* mutational carriers. Therefore we will continue our ongoing effort to collect specimens at our institute prospectively. A new protocol is planned for submission to the UCLA IRB for future tissue procurement and analysis for continuation of this project.

TASK 2: Assessment of frequency of *p53* gene mutations in *BRCA*-linked ovarian tumors identified in TASK I (months 6 - 36).

Task II is also relevant to Objective I of the proposal. In order to assess the frequency of *p53* mutations in *BRCA*-linked ovarian cancer, we first developed DNA sequence reactions to study mutational frequency. Conditions have been optimized for each of the PCR reactions required for sequencing runs using genomic DNA extracted from tumor tissues obtained from the Core Ovarian Tumor Facility. We anticipate being able to complete the proposed sequence analysis in the coming months; however, pilot studies (and previously published studies) indicate that for increased accuracy in the analysis of *p53* DNA sequence, it will be necessary to microdissect tumor cells from each of the banked ovarian tumor samples in order to avoid missing *p53* mutations due to stromal cell contamination of tumor tissues which would contain wild-type *p53* and could confound results from PCR amplification from genomic DNA extracted from ovarian tumor tissue. Strategically, because of the relatively high frequency of *p53* mutation in this disease, it may be possible in select cases to complete sequence analysis of available tumor samples without microdissection, with microdissection being reserved for those cases which are found to be wild-type on the original sequence analysis. Using this strategy, false negative results may be avoided by confirming *p53* mutational status on microdissected tumor cells. Another group has recently reported analysis of *p53* mutational status on *BRCA*-linked ovarian cancers (Buller, et al., Clin Cancer Res. 2001 Apr;7(4):831-8.). These investigators have reported as very high frequency of mutations as we have hypothesized in this proposal. No unique types of *p53* mutations were identified as being specifically associated with a mutant *BRCA* genotype in this study. Further data are needed to confirm these

findings and it is hoped that our data will complement the observations already reported by Buller and colleagues on this issue.

TASK 3: Assessment of MDM2 expression in *BRCA*-linked and sporadic ovarian cancers using immunostaining techniques (months 1-24).

Analysis of MDM2 expression in the available Core Facility Tumor Bank is currently ongoing. In addition we have access to a new large tumor bank from Munich with long-term clinical follow-up. We have recently utilized this cohort to measure expression of UPA and PAI-1 to demonstrate the prognostic significance of these markers in ovarian cancers of different clinical stages (Konecny, et al., Clin Cancer Res. 2001 Jun;7(6):1743-9). We aim to develop an ELISA format assay suitable for measurement of MDM2 expression using these same samples. Such analysis would expand the number of available samples for analysis significantly. Development of a suitable assay for retrospective analysis of large tumor banks is ongoing at this time.

TASK 4: To determine whether expression of wild-type *p53* in *p53*-mutant, *BRCA*-mutant Cedars Sinai Ovarian Carcinoma Cells (CSOC) is sufficient to induce cell cycle arrest, induction of P21, and apoptosis.

This task addresses Objective III of the proposal. In this aim, we compare the biological effects of *p53* in *BRCA*-linked ovarian cancer cells to those which are wild-type for *BRCA* genes. Our preliminary data indicate that one of the most important determinants for response to adenoviral vector containing *p53* cDNA is in fact expression of the adenovirus receptor p46 hCAR. However, cells which are *p53* mutant and express hCAR all respond to *p53* transfection in terms of induction of apoptosis, including CSOC lines which are known to harbor *BRCA*-1 mutations. We have now cloned the cDNA for human 46kD hCAR and have developed expression vectors for this cDNA. Transfection of hCAR into hCAR-negative ovarian cancer cells allows further ability to transduce these lines with our *p53* adenoviral vector so that we can now evaluate response to *p53* adenovirus in most all of the available CSOC lines. This finding also may have implications for Project #3 in which adenoviral vectors have been proposed for transfection studies of ovarian surface epithelium *in vivo*.

To determine the clinical safety of *p53*-containing adenoviral vector delivered into the peritoneal cavity of women with refractory ovarian cancer, we conducted a phase I/II dose escalation study of *p53* adenovirus administered intraperitoneally (i.p.) for women with refractory advanced ovarian cancer. No maximum tolerated dose (MTD) was established as the protocol -defined DLT was not met. The doses we delivered ranged from 7.5×10^{10} to 7.5×10^{13} particles per i.p. infusion. The highest dose tested was limited by practical considerations including the i.p. delivery volume for multiple -day dosing regimens. Tolerance to the adenoviral vector was satisfactory with manageable toxicity. Aside from

fever, the toxicity profile, even with multiple cycles was similar to that reported previously for i.p. chemotherapy in general. Overall, 82.2% of the planned doses were delivered and this included 219 of 270 (81%) doses on the multiple -dose/multiple – cycle regimens.

Vector-specific gene transfer and mRNA expression of *p53* was seen at doses as low as 7.5×10^{10} particles/single dose and was frequently detected in patients that received 7.5×10^{11} particles /dose. It seemed desirable to increase the dose level and number of doses to a maximum based on the theoretical tumor burden within the peritoneal cavity and the need to maximize exposure of tumor cells to *p53*-containing adenovirus. Our preclinical modeling indicated that multiple fractionated doses of *p53* vector had greater efficacy than a single bolus injection.

Early concerns that the presence of serum neutralizing antibodies to the adenovirus might limit its effectiveness, particularly with repetitive exposure are not borne out by our results. Previous preclinical work with immunized rodents treated with intratumoral injection of an adenoviral vector expressing IL-12 had demonstrated minimal reduction in transfer efficiency. Despite the generation of increased antiadenoviral antibody titers to *p53* adenovirus in all treated patients, we were able to demonstrate *p53* transgene expression after multiple cycles of dosing. There was no obvious enhanced transgene expression in the two individuals who were treated at level 1 because of no demonstrable adenoviral immunity. Not all patients underwent sampling with each cycle of treatment, due to the invasive nature of laparoscopic biopsy procedures. Nonetheless, our data clearly show the presence of transgene expression in RNA isolated from both ascitic fluid and tumor biopsies. The alternative explanation of persistent, stable expression of *p53* over time is inconsistent with *in vitro* and *in vivo* preclinical observations.

For a single case, *in situ* PCR data confirmed gene transfer in tumor cells obtained at laparoscopic biopsy. It is not possible to determine the percent of tumor cells transduced because of variability in the size of the biopsies obtained and the variation in the depth of adenoviral penetration. For example, in the case of a 3-mm biopsy with 1 mm of penetration and 100% transduction to the level of penetration, one might infer 33% transduction efficiency. However, because the size of the lesion is unknown, the true transduction efficiency cannot be calculated. Similarly, a smaller (2 mm) biopsy from the same site would provide a different estimate of transduction efficiency. This important parameter cannot be estimated nearly as well in human clinical trials as it can be in cell culture, or in orthotopic animal models with smaller and more uniform lesions. Further *in situ* PCR studies are ongoing and will be the subject of a future report.

TASK 5: To determine the frequency of *p53* alteration in "normal" ovarian surface epithelium from *BRCA*-linked individuals who have undergone prophylactic oophorectomy.

This task addresses Objective II of the proposal. This aim was cited by reviewers as the most difficult to complete. We have recently identified and evaluated normal ovarian tissues from several samples from the Ovarian Tissue Core Facility which met criteria for this study. Using an immunohistochemical screen for aberrant accumulation of mutant *p53* protein, we have sought evidence of *p53* mutation in these otherwise normal tissues. Thus far we have not identified cases of *p53* alteration in the tissues thus far evaluated with this methodology. There are at least two limitations to this approach however. One is that ovarian surface epithelium is frequently denuded from the ovarian samples during tissue procurement and processing in the pathology laboratory so it may be difficult to find the cells of interest on the slides following *p53* immunostaining. Moreover, we know from study of malignant ovarian tissue that there can be false negative results from *p53* immunostaining resulting from truncation mutations of *p53* due to point- or frameshift-mutations resulting in premature stop codons. Therefore it is anticipated that further study of these tissues may be required using microdissection followed by DNA extraction, PCR amplification, and sequence analysis for *p53*.

Project #3, Manipulation of Genes in the Ovarian Epithelium of Mice

Timothy F. Lane, PhD, Principal Investigator

Without models to test ideas about the initial stages of ovarian cancer, the task of identifying relevant markers and relevant targets for therapy becomes a daunting search for a needle in a haystack. One problem is the lack of a genetically defined animal model of epithelial ovarian cancer that can be used to test genes and gene pathways for their involvement during disease development. Techniques have now been developed for introducing genes into mice and expressing them in virtually any tissue type. Such an approach has led to major advances in our understanding of genes involved in a large number of other cancers. Successful demonstration of the utility of such an approach will make it possible to test the involvement of virtually any gene in ovarian cancer progression, and would be a major advance to the field. The strategy we proposed was to test the ability of wild type and modified adenovirus to deliver genes to normal ovarian epithelial cells *in vivo* with the idea that we could then use the *cre-lox* system to activate or delete genes of interest in a tissue specific fashion.

TASK 1: To establish the efficacy of Ad5-cre delivery to the ovarian epithelium (months 1-24)

The experiments specified in Task 1 of our Statement of work have progressed nicely. As specified in Task 1, we used a recombinationally activated gene cassette that would allow the production of a β -galactosidase gene only in cells expressing *cre* recombinase; this cassette is referred to as RABE. Several experiments have been carried out *in vitro* showing that the components of the system work well in cultured primary epithelial cells, and this allowed us to move into work on RABE transgenic mice. To date, we have injected Ad-cre into the ovarian capsule of 4 RABE female mice. We have looked for expression of β -galactosidase at 8 hrs (2 mice) and 24 hrs (2 mice). β -galactosidase positive cells were identified only in the 24 hr time point and transduction appeared to be rather inefficient. We are currently producing more concentrated viral stocks in the hopes that poor infection rates can also be overcome with higher titers.

A complication was also quite evident from these experiments. Adenoviral injections resulted in recruitment of a lymphoid infiltrate into sites of injection. We may request additional funds to document the cell types involved, but will likely switch to our proposed alternative strategy of transplanting the ovaries to avoid the need for exposure of animals to large amounts of virus.

We are also generating more mice to try the alternative strategy proposed for infecting the cells (ovarian transplants). This has been delayed because some of the mice became contaminated with a murine virus (MPV) and had to be destroyed. In year 2, we reestablished a clean colony of RABE mice, but continued to have problems with the RABE transgenic line due to MPV infection and eventually decided to discontinue the line. In order to replace the line we have obtained a ROSA26 (129s-gtrosa 26) from Jackson Labs which should be suitable. These mice are obtained in July and the colony has now been expanded to a useable size for our experiments. This has been a setback, but we hope to rapidly progress through the studies that were started in year one. Specifically, we have learned that direct injection of Ad5 particles is impractical due to immune infiltrate into the peritoneal cavity. Thus, we will be focusing our efforts on the more time consuming alternative strategy of infecting ovaries dissected from transgenic donors and then reimplanting them into host females. We have tried several pilots of this strategy and have encountered the following difficulties: 1) it is very difficult to manipulate mouse ovaries without removing the ovarian epithelium. 2) we were not able to use our standard FVB or 129vEv mice as recipients of ROSA ovarian transplants due to tissue rejection. These difficulties were not unexpected and will be overcome through use of syngeneic ROSA 129 hosts and improved skill in transplantation. Establishment of RA-DNp53 animals are scheduled to start at the end of November and the RA-herB2 will be injected in January

2003. Constructs for the *p53* were delayed because of the MPV infection in our colony. This infection has now been eliminated and we are scheduling all transgenic injections to proceed very rapidly.

TASK 2: To establish the ability of Ad5-*cre* delivery to delete genes from the ovarian epithelium (months 1-28)

The experiments specified in Task 2 are currently behind schedule due to a backlog in the availability of floxPTEN mice from Dr. Wu. As with our RAGE mice, the floxPTEN colony was dealt a setback from MPV infection. Thus we will be focusing our efforts on the more time consuming alternative strategy of infecting ovaries dissected from transgenic donors and then re-implanting them into host females. We have tried several pilots of this strategy and have encountered the following difficulties: i) it is very difficult to manipulate mouse ovaries without removing the ovarian epithelium. ii). we were not able to use our standard FVB or 129SvEv mice as recipients of ROSA ovarian transplants due to tissue rejection. These difficulties were not unexpected and will be overcome through use of syngeneic ROSA129 hosts and improved skill in transplantation.

We have learned that direct injection of Ad5 particles into immunocompetent mice is impractical due to immune infiltrate into the peritoneal cavity. We proposed to focus our efforts on isolating mouse ovarian epithelial cells and infecting them *in vitro* in preparation for transfer. Because of the issues with infection *in vivo*, we have chosen to pursue development of *in vitro* systems as proposed as an alternative strategy in our original application. We have developed a method of isolating mouse ovarian surface epithelial cells (MOSE) and find that they are efficiently infected by our retroviral constructs. Since it appears that transplantation will be required to carry out the proposed experiments, we believe it is impractical to pursue Ad delivery of *cre* and have instead established a collaboration with Thomas Hamilton, PhD, (Fox Chase Cancer Center, PA) who had recently established a transgenic system that is based on keratin 19 promoter. We have will transfer our remaining effort into placing our inducible *cre* recombinase construct into this promoter and establishing a transgenic line.

TASK 3: To establish the ability of Ad5-*cre* delivery to activate over-expression of transforming oncogenes in the ovarian epithelium (months 12-36)

TASK 4: To establish the ability of Ad5-*cre* delivery to activate over-expression of dominant negative anti-oncogenes in the ovarian epithelium (months 24-36)

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The experiments specified in Tasks 3-4 rely on development of plasmid vectors and transgenic mice in Task 3. Following problems with Adenovirus administration to immuno competent mice (outlined in previous reports) we abandoned the adenovirus strategy originally proposed. This update presents background and preliminary data that we developed to test the effect of progesterone receptor function in a mouse model of ovarian cancer. The preliminary data demonstrate a rationale for studying progestin action in the ovarian epithelium and demonstrate our commitment to developing mouse based systems to study ovarian cancer progression, as originally proposed in this application (see attached supplement, pages 29-45).

KEY RESEARCH ACCOMPLISHMENTS:

Ovarian Tissue and Clinical Database Core Facility

- Collected and snap froze surgical specimen from 152 Ovarian cancer patients
- Collected and snap froze surgical specimen from 110 patients with benign ovarian of which 29 were from patients with family history of ovarian cancer or *BRCA1* or *BRCA2* heterozygotes
- Collected blood and isolated serum and genomic DNA from all patients that donated surgical tissues
- Established primary cultures from 55 malignant ovarian tumors and 40 normal ovaries
- Cryopreserved primary cultures from 30 malignant ovarian tumors and 24 normal ovaries
- Provided all material requested to the 3 projects outlined in the Genetic Definition and Phenotypic Determinants of Human Ovarian Carcinomas Project
- Provided resources to the ovarian cancer scientific community
- Expanded and enhanced the Ovarian Tissue and Clinical Database Core Facility electronic database

Project #1, Molecular Biomarkers in Ovarian Cancer

- Cloned genes that are differentially expressed in ovarian cancer cells using cDNA-RDA.
- Sequenced cloned DNA fragments to identify 160 HOSE and 95 CSOC specific genes
- The cloned DNA fragments were used to fabricate a high density DNA arrays. These arrays were interrogated with cDNA probes from 15 different HOSE and CSOC cells to identify 46 HOSE-specific and 14 CSOC-specific genes that exhibited at least 2.5-fold difference in expression level
- Constructed an ovarian epithelial cell cDNA library
- We carried out a gene-expression profiling study using 10 HOSE and 22 CSOC samples
- We have generated a high quality anti-periostin antisera to study the expression of periostin in ascites and sera
- We have established immortalized ovarian epithelial cell lines by transducing the catalytic subunit of telomerase (hTERT) and/or HPV-16 E7

Project #2, Interactions Between *BRCA1* and *p53* Mutations in Human Epithelial Ovarian Carcinogenesis

- Identified *BRCA1*-linked ovarian cancers in the Ovarian Tissue Core Facility
- Established optimal conditions for each of 10 PCR reactions required for amplification of genomic *p53* sequence. We have confirmed the validity of this approach by sequencing *p53* from MDA-MB-231 cells which are known to harbor a specific *p53* mutation
- Identified a large cohort of both sporadic and *BRCA*-linked ovarian tumors suitable for MDM2 expression analysis
- Discovered an association between expression of the human coxsackie and adenovirus receptor and adenoviral vector transduction efficiency in ovarian cell cultures
- Elucidated anti-adenovirus antibodies as an inhibitor of adenoviral vector transduction in human malignant ovarian cancer ascites specimens
- Procured the first 8 samples of "normal ovaries" from known *BRCA1* mutation carriers. We will seek evidence of *p53* gene mutation in these premalignant specimens

Project #3, Manipulation of Genes in the Ovarian Epithelium of Mice

- Created a new construct that carries the *cre* recombinase under the control of the k18 promoter
- Tested the ability of this construct to mediate recombination in mouse ovarian epithelial cells in culture
- Generated Ad5-*cre* under the control of standard adenoviral promoter elements for pilot work *in vitro* and *in vivo*

REPORTABLE OUTCOMES:

Ovarian Tissue and Clinical Database Core Facility

1. A well endowed human tissue and serum repository for normal and malignant ovarian tissues and corresponding serum and germline DNA.
2. The development and preservation of primary cultures of normal and malignant human ovarian epithelial and stromal cells.
3. The Ovarian Cancer Laboratory and Clinical Database, linking laboratory specimens and results with patient demographic, epidemiologic and clinical data.

Project #1, Molecular Biomarkers in Ovarian Cancer

1. Matei D, Graeber T, Karlan BY, and Chang DD: Gene-expression profiling in normal and malignant ovarian epithelia. *Oncogene* 21:6289-6298; 2002 (previously submitted).
2. Gillian L, Matei D, Fishman DA, Gerbin CS, Karlan BY and Chang DD: Periostin secreted by epithelial ovarian carcinoma is a ligand for avb3 and avb5 integrins and promotes cell motility. *Cancer Research* 62:5358-5364; 2002 (previously submitted).

Project #2, Interactions Between *BRCA1* and *p53* Mutations in Human Epithelial Ovarian Carcinogenesis

1. Buller RE, Runnebaum IB, Karlan BY, Horowitz JA, Shahin M, Buekers T, Petruskas S, Kreienberg R, Slamon D and Pegram M: A phase I/II trial of rAD/p53 (SCH 58500) gene replacement in recurrent ovarian cancer. *Cancer Gene Therapy* 9:553-566; 2002 (previously submitted).
2. Buller RE, Shahin MS, Horowitz JA, Runnebaum IB, Mahavni V, Petruskas S, Kreienberg R, Karlan B, Slamon D and Pegram M: Long term follow-up of patients with recurrent ovarian cancer after Ad p53 gene replacement with SCH 58500. *Cancer Gene Therapy* 9:567-572; 2002 (previously submitted).
3. Konecny G, Untch M, Pihan A, Kimmig R, Gropp M, Stieber P, Hepp H, Slamon D, Pegram M: Association of urokinase-type plasminogen activator and its inhibitor with disease progression and prognosis in ovarian cancer. *Clin Cancer Res* 7(6):1743-1749; 2001 (previously submitted).

Project #3, Manipulation of Genes in the Ovarian Epithelium of Mice

1. Developed an adenoviral vector expression *cre* recombinase and a variant with the K18 promoter.
2. Determined that adenoviral delivery of *cre* in immunocompetent mouse peritoneal cavity is impractical due to immune reactions at the site of injection.

CONCLUSIONS:

This program project is a multidisciplinary collaboration aimed at elucidating genetic alterations that contribute to human ovarian carcinoma with an eye towards identifying useful targets for early ovarian cancer detection and prevention. Towards these ends, the **ovarian tissue core facility** has banked over 160 surgical specimens, including 96 ovarian carcinomas and 71 benign ovaries, of which approximately one third are from women with a family history of ovarian cancer or a known *BRCA* mutation. Furthermore, the Core's tissue resources are linked to clinical, demographic, and epidemiologic data that allows us to make clinical correlations with our laboratory findings. The Core together with the Clinical Database will support all avenues of ovarian cancer research including those directed toward understanding the basic biology, etiology, genetic influences, prevention and therapeutic developments. In **Project 1**, representational difference analysis was used to identify 46 genes significantly overexpressed in normal ovarian epithelium and 14 specific genes overexpressed in ovarian cancer cells. Using these subtractive cloning techniques as well as a chip based expression profiling approach, we have successfully identified target genes that could be developed into biomarkers for ovarian cancer. In addition, the newly established immortalized ovarian epithelial cell lines will be used to study the functions of the genes that are differentially expressed in cancer-derived ovarian epithelial cells. **Project 2**, has established the necessary techniques and identified the *BRCA1* mutation associated ovarian tissues required to understand the functional interaction and contribution of *p53* and *BRCA1* to ovarian epithelial transformation. In addition, as a byproduct of these studies, we discovered that adenoviral gene transfer may only be efficiently accomplished in ovarian cells which express a gene called hCAR (human coxsackie and adenovirus receptor). This observation may have far reaching implications for patients undergoing gene therapy for ovarian cancer using adenoviral vectors. **Project 3**, has focused efforts at creating the necessary viral constructs for the proposed experiments aimed at establishing a murine human ovarian cancer model. A new construct carrying the *cre* recombinase under the control of the K18 promoter has been tested to establish its ability to mediate recombination in mouse ovarian epithelial cells. Work on general application of adenovirus into various mouse tissues has demonstrated complications. We will focus future efforts on using our ability to transplant ovaries to overcome this problem. We have determined that adenoviral delivery of *cre* in immunocompetent mice peritoneal cavity is impractical due to immune reactions at the site of injection. Recent development of a viable surface epithelial promoter for standard transgenics has made the adenoviral approach less appealing. We thus plan to switch over to the K19 promoter in collaboration with the lab that developed it as a viable alternative.

“So what?” In order to reduce the unacceptably high mortality rate associated with ovarian cancer, diagnostic modalities which can reliably detect early stage ovarian cancer and preventative strategies to diminish the number of new cases must be discovered. This Program Project has undertaken a multi-faceted approach to the ovarian cancer problem. Using the human ovarian specimens and clinical correlates provided by the core facility, new genes will be identified in Project 1 to serve as targets for detection, prevention, and/or therapy; functional interactions between important known genes, *BRCA1* and *p53*, will be elucidated in Project 2 and shed light on the molecular etiology of ovarian cancer; and a murine animal model to test these findings and others *in vivo* will be established in Project 3. At the conclusion of this program project, we will be closer to our goal of rationale rather than empiric approaches to ovarian cancer prevention, early detection, and therapy.

REFERENCES:

1. Matei D, Graeber T, Karlan BY, and Chang DD: Gene-expression profiling in normal and malignant ovarian epithelia. *Oncogene* 21:6289-6298; 2002 (previously submitted).
2. Gillan L, Matei D, Fishman DA, Gerbin CS, Karlan BY and Chang DD: Periostin secreted by epithelial ovarian carcinoma is a ligand for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and promotes cell motility. *Cancer Research* 62: 5358-5364; 2002 (previously submitted).
3. Buller RE, Runnebaum IB, Karlan BY, Horowitz JA, Shahin M, Buekers T, Petruskas S, Kreienberg R, Slamon D and Pegram M: A phase I/II trial of rAD/p53 (SCH 58500) gene replacement in recurrent ovarian cancer. *Cancer Gene Therapy* 9:553-566; 2002 (previously submitted).
4. Buller RE, Shahin MS, Horowitz JA, Runnebaum IB, Mahavni V, Petruskas S, Kreienberg R, Karlan B, Slamon D and Pegram M: Long term follow-up of patients with recurrent ovarian cancer after Ad p53 gene replacement with SCH 58500. *Cancer Gene Therapy* 9:567-572; 2002 (previously submitted).
5. Konecny G, Untch M, Pihan A, Kimmig R, Gropp M, Stieber P, Hepp H, Slamon D, Pegram M: Association of urokinase-type plasminogen activator and its inhibitor with disease progression and prognosis in ovarian cancer. *Clin Cancer Res* 7(6):1743-1749; 2001 (previously submitted).

Genetic Definition and Phenotypic Determinants of Human Ovarian Carcinomas
Beth Y. Karlan, MD, Principal Investigator
Award Number: DAMD17-99-1-9503

APPENDICES

1. Project #3 - Supplement
Manipulation of Genes in the Ovarian Epithelium of Mice
Timothy F. Lane, PhD, Principal Investigator

Project #3 – Supplement
Manipulation of Genes in the Ovarian Epithelium of Mice
Timothy F. Lane, PhD, Principal Investigator

Background:

Cancer develops through accumulation of mutations that frequently target regulatory pathways in cell proliferation and apoptosis. Because mutations are fixed into DNA during replication, mutation rate is proportional to the number of cell-cycle events and, proliferating cells are more likely to acquire and fix mutations in their DNA. The ovarian surface epithelium (OSE) is a tissue where cycles of wounding (ovulation) induce proliferation and tissue repair. This cyclic tissue-repair process exposes the OSE to enhanced risk of somatic mutations. Epidemiological data indicates that ovarian cancers increase with age and ovulatory cycles, and decrease with pregnancy and/or anti-conceptive agents that suppress ovulation. This reinforces the hypothesis that reduction in the number of cell-replicating events prevents the malignant state. In fact, the incidence of ovarian cancer is highest after menopause, interestingly women who opt for hormone replacement therapeutics that contain progesterone consistently demonstrate reduced ovarian cancer rates. Therefore, ovarian cancer is frequently considered a disease of aging and is critically dependent on ovarian history, life style/history and the management of hormonal balance in young and aging women.

Current challenges in ovarian cancer are identical to those faced by other cancers and relate to prevention, identification of markers of various cancer subtypes, earlier detection and development of more efficacious and targeted treatment. However, unlike other cancers, basic and translational research in this cancer type is significantly impeded by the lack of appropriate animal models that mimic the onset and progression of disease. It is clear that the focus in the basic sciences for this disease should be to: develop adequate models for ovarian cancer, further identify the key genes most commonly targeted by this particular disease, gain a concrete knowledge on the basic cell biology of ovarian epithelium to provide guidance on how to reduce disease.

Our laboratory is committed to the study of genes implicated in epithelial malignancies in women, specifically in relation to studies of tumor suppressor genes and generation of animal models. We have recently gathered results that, in combination with published studies, indicate PR actively participates in apoptosis of ovarian epithelial cells, and that this function is modified or absent in at least a subset of ovarian epithelial-derived cancers. More specifically, we have found that intact mouse ovaries showed apoptotic figures in the OSE when exposed to triphasic administration of E2 and P, and in vitro studies demonstrate a progesterone-dependent susceptibility to apoptosis in proliferating OSE. These results shaped the central hypothesis of this application that: PR function is protective against the incidence and development of gynecological ovarian cancer through its normal function in pre-malignant ovarian surface epithelial cells. These effects include regulation of apoptosis and, the response of cells to proliferation during post ovulation induced repair.

We have designed two specific aims to test this hypothesis at the molecular, cellular and whole-animal level.

AIM 1: To generate transgenic mice that will allow analysis of progesterone receptor contribution to growth regulation of ovarian surface epithelial cells during physiology and disease.

AIM 2: To ascertain the contribution of PR signaling in cell-cycle progression and sensitivity to apoptotic agents. Using primary cultures of ovarian epithelial cells, we propose

to expand upon our findings that PR activation sensitizes cells to apoptosis and determine the contribution of genomic versus non genomic activities of PR to each of these processes.

Our goal is to take advantage of recent advances in transgenic approaches to ascertain the contribution of PR to gynecological cancers using mouse genetics and to further dissect the molecular pathway of PR function in apoptosis using a combination of cell and molecular approaches. The work is timely, because of i. the very recent establishment of ovarian epithelial targeting promoters, ii. the growing epidemiological evidence that progestins (in contraceptives and hormone replacement therapy (HRT)) dramatically lower ovarian cancer rates potentially through an apoptotic effect on the OSE, and iii, evidence from HRT for a role of progestins in decreased ovarian cancer risk in post menopausal women. We view this proposal as a high risk/high impact. Clearly, generation of the animal model proposed will enable us to study PR signaling in OSE and offer a benefit to the scientific community beyond the boundaries of this application (as we will naturally share the reagents). As a parallel approach, the proposed in vitro and in vivo studies will enable the molecular dissection of PR and its contribution to apoptosis in ovarian epithelium.

BACKGROUND AND SIGNIFICANCE:

Progestins reduce ovarian surface epithelial cancer: Progestin containing anti-conceptive (contraceptive) therapies have been touted as one of the most effective means of reducing lifetime ovarian cancer risk in American women (Jensen and Speroff 2000). However, the mechanism of this effect, and the role of the ovarian epithelium, is not understood (Auersperg et al. 2001).

Along with parity, oral contraceptive (OC) use has consistently been associated with a decreased risk of ovarian cancer (Whittemore et al. 1992; Weiss et al. 1996). Three or more years of OC use, reduces the risk of developing epithelial ovarian cancer by 30%-50% (Weiss et al. 1981; 1987; Whittemore et al. 1992; Rosenberg et al. 1994). This 'benefit' increases with continued use and appears to be independent of linkage to familial ovarian cancer predisposing mutations in either BRCA1 or BRCA2 genes (Risch et al. 1983; Whittemore et al. 1992; Narod et al. 1998). Original formulations with high estrogen content and later formulations with reduced estrogen reduce ovarian cancer by reducing ovulation (a 'risk' factor for ovarian cancer). However, recent evidence that progestins regulated OSE responses to ovulation (Cancer and Steroid Hormone Study 1987), the lack of genetically tractable models of ovarian cancer has meant that the target tissue of individual agents within these formulations has been difficult to study. Given that ovulation is suppressed (Crosignani et al. 1996), and that breast and endometrial tissues are targets of progestins, these results paint a very narrow and nonspecific view of the tissues affected (Auersperg et al. 1998). In fact, the role of progesterone and its agonists on ovarian cancer progression remains controversial (Jolles et al. 1983; Kauppila et al. 1983). The problematic nature of interpreting the effects of progestins stems from heterogeneity in the response of ovarian cell lines to this agent and to conflicting accounts on the efficacy of steroid hormones and hormone antagonists on established clinical cases. Some ovarian cancer cell lines are inhibited by progesterone (Hu and Deng 2000; McDonnell and Murdoch 2001), while progestin antagonists inhibit others (Rose et al. 1996; McDonnell et al. 2001). In addition, manipulation of progestins has shown little consistent benefit in established clinical cases (Nash et al. 1989; Munstedt et al. 2000). The effect of progestin exposure late in life has also been inconclusive but is potentially more supportive for a direct role of progestins on ovarian cancer initiation. Specifically, hormone replacement therapy (HRT) formulations that lack progestins clearly increase ovarian cancer risk (estrogen alone), and inclusion of progestins seems to block this, corroborating a role for progestins in inhibiting the onset of ovarian cancer. Supporting this is a consistent series of studies demonstrating that estrogen and progesterone receptor positivity predicts a favorable tumor biology and long-term survival (Munstedt et al. 2000). Due to the fact that the target tissue may include multiple cell types and

organ systems, it is possible that the role of individual modifying agents, such as hormonal exposure, will be difficult to analyze in women.

Is PR a tumor suppressor gene for OSE: The most straightforward evidence that a gene has tumor suppressor function is to identify loss-of-function alleles in the tumor of interest. Interestingly, there is presently considerable debate in the literature as to whether PROGINS, a variant PR allele first identified as an Alu RFLP, in Australian ovarian cancer patients (Wang-Gohrke et al. 2000; Runnebaum et al. 2001; Whittemore and McGuire 2002). Such an excess prevalence of homozygous carriers among patients is consistent with a recessive role for the PROGINS allele in ovarian cancer, and would strongly support the rationale for our current application. The Ovarian Cancer Problem: Approximately 90% of ovarian cancers derive from the ovarian surface epithelium (OSE), yet the biology of the OSE is poorly understood. More than 26,000 new cases of ovarian cancer are identified each year in the United States, with almost 75% of these malignancies in advanced stages at time of diagnosis. Ovarian carcinoma is also the leading cause of death from gynecological malignancies in Western industrialized countries (Auersperg et al. 1998). Early-stage disease has a cure rate of up to 90%, but rates for advanced disease are dismal, approaching 5-20%.

Progesterone Receptor: Progesterone receptor (PR) is a member of a family of nuclear receptors capable of regulating gene expression upon binding the hormone ligand. The effects of progesterone are mediated by two receptor proteins (PR), termed A and B, that arise from a single gene and act as ligand-activated transcription factors to regulate the expression of reproductive target genes (Conneely and Lydon 2000). At present most of the downstream molecular and cellular mechanisms by which progesterone exerts its effects are unclear; however, the progesterone signal is known to be mediated initially by the progesterone receptor (PR). In most tissues studied, the PR is induced by ovarian estrogen via the estrogen receptor (ER), thereby implying that many of the observed reproductive physiological responses attributed to PR could conceivably be due to the combined effects of progesterone and estrogen. Recently it has also been demonstrated that P is able to rapidly activate a variety of signaling pathways in a transcriptionally independent (non-genomic) manner (Boonyaratanakornkit et al. 2001). This landmark finding opened a host of possibilities to explain rapid effects of P not simply understood by transcriptional regulation.

Role of Progesterone in Aging and Development: Progesterone plays a central coordinate role in regulating reproductive events associated with the establishment and maintenance of pregnancy including ovulation, uterine and mammary gland development and tumorigenesis, and neurobehavioral expression associated with sexual responsiveness. Thus, PR is considered a transcription factor, which is required for normal development and morphogenesis of reproductive organs (Conneely et al. 2000). In female PRKO mice, the ovaries achieve nearly normal size and have recognizable oocyte development. However, oocytes fail to mature past the stage of primary follicles and ovulation is absent. PRKO mice do have an intact OSE (see preliminary data) but the failure to ovulate means this animal is unlikely to provide a model of ovarian cancer, a disease that is largely dependent on cycles of ovulation and tissue repair.

The Ovarian surface epithelium (OSE) and responses to ovarian steroids. The OSE is a sheet of squamous to cuboidal mesothelial cells with pluripotential capacities, retaining both epithelial and mesenchymal potential. While the development of ovarian tumors is dependent on sex steroids, the contribution of these steroids to OSE growth regulation is controversial. Studies on primary monkey OSE have been interpreted to suggest that progestins have little growth stimulatory activity on OSE (Murdoch 1998a; Murdoch et al. 2001). The mechanistic relationship between increased ovulation and carcinogenic changes in the OSE cannot be elucidated without a better understanding of the

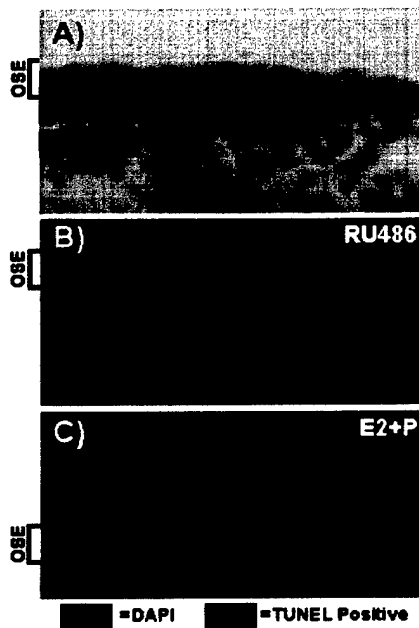
mechanisms regulating proliferation of OSE cells in response to normal hormonal stimuli. Recent progress in identifying ovarian epithelial specific promoters: Ovarian cancer lags behind mammary, prostate, cervical, lung and pancreatic cancers, all of which have viable transgenic animal models. The lack of a genetically tractable model of ovarian cancer hampers the development of our understanding of the basic cell biology of this disease and eliminates one useful model for preclinical drug screening for this particular cancer type (Tanyi et al. 2002). The KRT5 promoter does provide specific epithelial targeting of the transgene but this promoter is typically active in basal keratinocytes and it is not clear if the expression seen the Orsulic paper will be reproduced in other transgenic animals. The combination of KRT5 driven transgene expression, followed by transplantation of the ovary, offers a potentially viable strategy (epithelial targeting vector followed by transplant) for studying epithelial signaling pathways in the ovary (Orsulic et al. 2002).

We believe that a combination of the simple epithelia specific human keratin 19 (CK19) promoter and ovarian transplantation will permit the development of an OSE-specific mouse model, a species in which much progress has been made in understanding the genetic basis of disease. We believe that the ability to analyze the contribution of genes to the pathway cellular transformation will become increasingly useful as more genes are implicated in ovarian cancer progression (a specific goal of AIM1). In summary, two facts have fundamentally hindered progress in identifying genes involved in the onset of ovarian cancer. First, pre-cancerous lesions (and early tumors) are difficult to detect and subject to genetic analysis. Second, there are no animal models by which to study the contribution of genes to the precancerous, or transformed, lesion. Mice provide an accessible and genetically defined model in which it is possible to manipulate gene expression in inbred animals and then to study the effects of genetic background, environment, or treatment regimens on the behavior of the engineered cells in vivo. A primary advantage is the ability to study the effects of candidate genes on each stage of the transformation process and in the context of a functional immune system. We propose to take advantage of recent developments in 'tissue specific gene targeting' to generate mouse model of progesterone receptor function that will be relevant to the study of ovarian cancer.

PRELIMINARY STUDIES: The impact of PR signaling to the physiology of the female reproductive organs was determined by genetic ablation through homologous recombination studies. Mice that lack PR (PRKO mice) display pleiotrophic pathology including failure to ovulate and hypoplasia of the mammary gland. However, if indeed lack of PR is permissive for the development of tumors, then why PRKO mice have not been reported to have increased incidence in breast and ovarian cancer? And, therefore, why engage in the present line of investigation? At least two points can be offered to explain the lack of tumor incidence in the PRKO mice: PR signaling during development is essential for maturation of reproductive organs and consequently, when absent from conception, these organs are indeed immature, and/or developmentally arrested, and cannot be compared to fully mature and responsive organs that have been exposed to normal PR signaling. PR function in the adult is only protective (not causative); therefore unless an oncogenic insult is present, these animals will not develop cancer. Crosses between the PRKO animals and transgenic mice housing oncogenes have not been done. However, the fact that PRKO mice fail to ovulate and have defective hormonal and behavioral functions, make them a poor choice for these studies.

Conditional and cell-specific knock-outs for PR can easily eliminate those issues. In Aim 1 we propose to generate a conditional OSE targeted cre recombinase allele in mice (CK19creERT2). The resulting animal will be crossed with floxExon1-PR animals (described below). In combination with ovarian transplantation into non transgenic hosts, this strategy will generate ovarian epithelial-PR null animals at any time during the life-span of the transgenic animal dependent on the presence of tamoxifen for 4-7 days. Experiments can be initiated several weeks after treatment to rule out any concerns related to circulating levels of tamoxifen. Nonetheless, before engaging in these studies is important to undertake

detailed analysis on the expression of PR in normal ovaries.



◆ **FIGURE 1-** Mouse OSE express PR and show increased TUNEL positive nuclei in response to a cyclic administration of E2 and P. Panel A, Mouse ovaries were dissected from PRKO animals expressing a β -galactosidase cassette inserted into the PR locus. In ovarian sections, PR locus activity (BLUE) is visible in scattered ovarian surface epithelial cells (OSE) as well as in cells underlying this epithelium. Cells are counter stained to show nuclei (RED). Panels B and C. Wild type female mice (8weeks) were treated with low dose Estradiol and progesterone in the presence (B) or absence (C) of RU486. After 3 cycles of 3-day treatments followed by 1-day rest, ovaries were isolated and processed for TUNEL positive nuclei (ORANGE) and counter stained (BLUE) to reveal nuclei. Results: TUNEL positive dots were present in most OSE nuclei and were present in some, but not all sub epithelial nuclei.

A combination of antibody studies and PR-lacZ (knock-in of the lacZ in the PR locus) were initiated. Our preliminary data is consistent with some published studies using antibodies and indicates that PR is expressed by the ovary, selectively by granulosa cells (from large primary follicles), scant stroma, and ovarian epithelium. Expression by the ovarian epithelium is associated with sites of large primary follicles and enhanced/focused in focal points, possibly sites of pre- and post-ovulation given its association with large primary follicle. Figure 1A shows expression of lacZ (in blue,) in a selective subset of ovarian epithelial cells. Positive cells can also be seen in the stroma and underlying follicle. As reported in the literature (Murdoch et al. 2001), we also confirmed that exposure to prolonged exposure to E2 and P (to mimic oral contraceptives) predisposes/ enhances apoptosis in the ovarian surface epithelium (Fig. 1C). Interestingly, presence of RU486, a progesterone antagonist, blocks the apoptotic events (Fig. 1B).

To further pursue these observations, we isolated ovarian epithelial cells from wild-type and PRKO mice and manipulated expression of PR in vitro to determine the specific effect of PR signaling on cell cycle regulation in the presence or absence of DNA damage. Isolation of surface epithelial cells from mouse ovaries has not been reported. We devised a protocol that reproducibly yields pure populations of OSE cells for in vitro studies from few ovaries. Briefly, ovaries are dissected and incubated for 10 min in an FITC-conjugated vital lipophilic compound (Molecular Probes cat#7778), washed 4 times for 5 min and exposed to mild digestion under agitation. The cell suspension is then filtered in nylon meshes and sorted by FACS.

Figure 2 shows the specificity of labeling of the compound for the surface epithelium (Fig 2A,B), the unsorted populations of cells (Fig. 2C and D), and the resulting epithelial cultures after sorting (Fig 2E, F). Note that expression of strong nuclear PR is observed in the cultured cells. Using this protocol, we have been able to isolate ovarian epithelial cells from PRKO mice and wild-type littermate animals (Fig 3). In our hands these primary epithelial cultures can be maintained for several weeks under culture conditions and can be propagated up to 3 times (1:3 ratio) without noticeable alterations in cell size or indications of senescence. After passage 4 cells undergo progressive senescence. Mouse OSE were isolated from wild type and PRKO mice and show similar abilities to

grow as contact inhibited clusters. Mouse OSE were isolated as before and stained with antisera to PR and β catenin. The isolated populations can be infected with PR adenovirus to either achieve homogeneous expression of PR, rescue expression in the null cells with wild-type or mutated forms of PRA, B or both. Figure 4 shows adenoviral expression of PR in PRKO-derived cells.

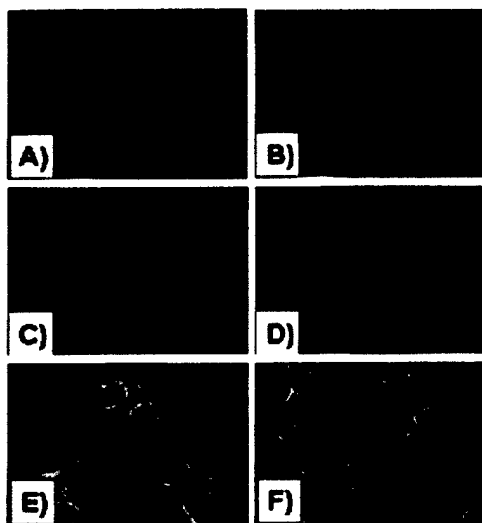


FIGURE 2- Isolation of mouse OSE and localization of PR in non-contact inhibited regions in clusters of freshly isolated cells. Panels A and B. Mouse ovaries were dissected and immersed in FITC-labeled lipophilic dye and then sections were made to demonstrate specificity of labeling. FITC (GREEN) AND DNA (BLUE). Panels C and D, Similarly labeled ovaries were then placed in tissue disruption buffer containing trypsin, collagenase, and RNase for 4 hrs to yield single cells and clusters of cells. Single cell suspensions were then sorted with a FACScan instrument. Panels E and F. FITC labeled cells were plated and cultured in DMEM+2%FCS+insulin+EGF +Dexamethasone for 1 week before immuno-staining for Progesterone receptor (RED) and β catenin (GREEN)..

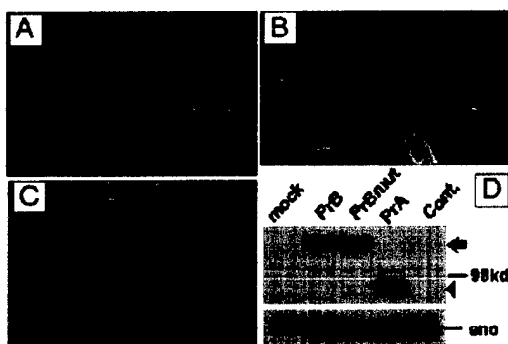


FIGURE 3_ Isolated OSE are efficiently infected with adenovirus (Ad) expression PR-A and PR-B isoformss.

Panel A) Absence of PR (RED) from mock infected PRKO derived OSE. PRKO OSE after infection with Ad-PR-B expression vector (Panel B) or Ad-PR-A expression vector (Panel C). Panel D shows immunoblots of PRKO derived EC cell extracts following infection with various PR expression vectors. The upper blot is stained with the PR antibody, the lower blot with enolase as a loading control. Lane 1, mock infected (empty virus), 2, Ad-PRB virus, 3, Ad-PRB with a mutation in the DBD, 4, Ad-PRA virus, 5, uninfected control PRKO cells. Panels A, B, C were immuno-stained as in Fig 3. PR (RED) and β -catenin (GREEN).

These results support feasibility for the experiments proposed in Aim 2. Adenoviral vectors were a generous gift of Dr. Dean Edwards (Univ. of Colorado). Dr. Edwards will provide us with these constructs and several mutated forms (see enclosed letter of collaboration). The PR adenovirus used in Fig 4D, lane 3 is a DNA-binding mutant (DBM), able to activate src signaling, but unable to act as a transcriptional regulator as it is not able to bind to DNA (Boonyaratanakornkit et al. 2001). A second mutant able to act as a transcription factor, but unable to bind to src will also be made available to us and allow us to dissect the relative contributions of genomic versus non-genomic PR-signaling events in cell-cycle and apoptosis. To determine the effect of PR on cell-cycle regulation and apoptosis in vitro, we first employed a human ovarian cell line (PA1). The criteria for the selection of this particular cell line from the 8 ovarian cell lines screened included: 1) expression of endogenous PR (at low level, but comparable to primary cultures); 2) evaluation of functional PR parameters, such as translocation of PR to the nucleus upon exposure to P and transactivation of a PR-responsive element. These experiments will be repeated and expanded with primary cultures of murine ovarian epithelial cells from wild-type and PRKO mice and after rescued expression with PR adenoviral wild-type and mutated forms (proposed in Aim 2).

Exposure of PA1 to physiological levels of progesterone results in cell-cycle arrest in G2 and a 32% increase in apoptotic rate versus control (Not shown due to lack of space.). The effect of P on cell cycle and apoptosis was blocked by the progesterone antagonist RU486 in a dose-dependent manner. Thus, 1 μ M of P increases apoptosis by nearly 30%, addition of 0.2 μ M RU486 reduces this effect to 6%.

Unlike primary OSE, human ovarian epithelial cell lines express low, to undetectable, levels of immuno-reactive nuclear PR. Three ovarian cell lines PA1, A2780, and OvCar1065. were treated with vehicle, 1 μ M Progesterone, or 1 μ M RU486 for one hour before fixation and processing for immunofluorescence. Unlike freshly isolated mouse OSE, the cancer cell lines expressed negligible PR immunoreactivity. Staining was enhanced in all cell lines by either progesterone or RU486, but only PA1 cells showed significant nuclear relocalization of the receptor. A2780 cells showed negligible PR even in the presence of ligand and OvCar1065 cells showed enhanced cytosolic but no nuclear PR when exposed to ligand. A2780 cells are also resistant to P-mediated apoptosis. Reconstitution of PR expression, results in sensitization to apoptosis when in the presence of PR. The apoptotic response can be blocked by addition of RU486 (not shown with apologies).

Reviewers of this proposal might argue that the suppressive effect of PR on epithelial cell cycle and apoptosis has been reported. In fact, an exhaustive evaluation of the literature reveals that the effect of P on epithelial cell lines has been quite controversial with studies reporting stimulation and an equal number of studies reporting suppression of proliferation and/or an increase in apoptosis. Truthfully, in many of those studies the status of PR (expression and functional parameters) was not a consideration, making it difficult to ascertain the validity of the conclusions raised from the data. In addition, most of the studies were performed in cancer cell lines. Many of these cell lines have built up evasive responses to apoptotic signals by an accumulation of progressive mutational events. Thus, we feel that this question (P-effects on proliferation) has not been resolved. In addition, the compelling body of epidemiological data supporting a protective effect of P in gynecological malignancies offers a significant rationale to pursue well-controlled in vitro and in vivo studies and determine the function of P in normal

physiological and pathological states. We concur that utilization of transgenic mouse models brings in their own distinct caveats; in particular and foremost the issue of adequacy of murine species to answer questions related to human reproductive physiopathological states. Clearly the similarities between the species in relation to reproductive physiology are equally numbered by their differences. However, we feel that this is the approach with highest potential to yield meaningful information. In fact, in the last ten years, the most significant advances of our knowledge of biological function of proteins (reproductive system included) have come from manipulation of the mouse genome. Finally, we plan to use this animal model (conditional PR loss-of-function) as a platform for the introduction of pertinent oncogenes / LOH tumor suppressor genes by selective crosses using animals already at hand (BRCA1 anti-sense,

Lane et al., 2000, p53^{-/-}, myc) and answer questions related to the contribution of PR to cancer progression and aging. The discussion of these experiments is beyond the time-frame and resources of the present application, but is part of our long-term commitment to this project.

A possible improvement of cre-recombinase expression is the possibility to control the timing of action/ expression. Recent advances currently allowed the generation of constructs with possibility of conditional expression. Recently Feil and colleagues reported the generation of a cre-recombinase construct linked to a mutated form of the estrogen receptor unable to respond to estradiol, but able to bind to tamoxifen. Translocation of cre to the nucleus require ligand binding (tamoxifen) therefore, in the absence of tamoxifen the creER fusion protein remains in the cytoplasm as is functionally inert. While the use of tamoxifen is an added caveat to this model system, the levels required to activate the mutant protein are 10-20 times lower than that required to elicit physiological effects. Furthermore, based on our experience using this construct with our collaborator (Dr. Iruela-Arispe), only 4 days of treatment is required to effectively induce cre and lead to inactivation of the floxed allele in the cell type where the promoter is active (in the case of our collaborator, on endothelial cells).

We recently generated an endothelial targeting vector expressing creERT2 with our collaborator Dr. Iruela-Arispe. 5 founders are being mated against the ROSA26floxBgal reporter strain to assess inducibility.

A final piece of preliminary data relates to the status of the floxExon1PR animal, currently being generated in collaboration with Dr. Iruela-Arispe (see letter). A fragment of genomic DNA from 129/SvJ library was provided by Dr. John Lyndon (Baylor College of Medicine). This same fragment was used in the generation of the now standard knockout model of PR, the so called PRKO mouse (Lydon et al. 1995). To generate a conditional PRKO animal (floxExon1PR) we are introducing loxP sites flanking exon 1. The first loxP site was engineered successfully by introducing a floxed PGKneo cassette into the EcoRI site in intron 1. The PGKneo cassette provides positive selection in ES cells and is flanked by loxP sites both ends to permit excision of PGKneo. prior to injection of ES cells into blastocysts. This will eliminate concerns related to expression of neo in the PR locus. The second loxP will be placed in an EcoRI site in intron2.

The generation of a PR-floxed mouse will enable inactivation of the gene in a cell or tissue-specific manner and dependent on the expression of cre-recombinase. Aim 1 proposes to inactivate the PR locus in OSE with CK19-creERT2. A simple epithelial targeting promoter

based on the keratin 19 promoter (CK19) has been provided by our collaborator Dr. Thomas Hamilton (Fox Chase Cancer Research Center). Dr. Hamilton's lab has generated a non-inducible CK19-cre and characterize the pattern of cre expression using ROSA26flox. Expression is confined to simple epithelial cells throughout the animal including the OSE (manuscript in preparation). Mice expressing large T antigen under this promoter develop lung and other adenocarcinomas, indicating that an inducible system such as creERT2 and transplantation will be required to make an OSE specific model. Since all of the molecular reagents are at hand, no problems are expected with the constructs and we hope to begin validating the reagents for expression on primary OSE in the near future. The rationale for generation of creERT2 animals from the outset of this project is the problem with 'leaky' expression of in non OSE epithelium (peritoneum, lung, etc) and its possible effects on overall ovarian function. We argue that we need a fully functional ovary, in particular with regards to ovulation to pursue experiments related to ovarian cancer and we need to be confident that cancer tissue (if it develops) is from the OSE and not some other peritoneal source. We will assess expression by mating all founders with the ROSA26flox β gal reporter strain +/- tamoxifen treatment. Although we propose to use Dr. Hamilton's cre (constitutive) as a fall back position, we anticipate that the creERT2 will be an essential reagent for developing a truly OSE- specific model and for analyzing loss of PR after multiple rounds of ovulation as a model of OSE aging. We hope that preliminary data presented have conveyed the following points related to the rationale of this application: P modulates cell-cycle progression and apoptosis. The effect of P can be suppressed by RU486 and can be elicited by incorporation of PR in 'functionally-depleted' cell lines. Both pieces of data implicate PR as a mediator of the P-effects.

Feasibility: The use of mouse: much like in primates, PR is expressed by the murine surface ovarian epithelium in vivo (Fig 1A). Expression can be maintained in vitro with a similar degree of heterogeneity (Fig 2). Our laboratory has expertise in the isolation and propagation of primary cultures of ovarian epithelial cells (Fig 2 and 3) and have isolated both wild-type and PRKO primary ovarian epithelial cells (Fig 3). Reagents to manipulate expression of PR and alter PR functional responses to genomic and non-genomic pathways are at hand (Fig 4). We have experience with the generation of transgenic animals and evaluation of cre-expression (Fig 10). Reagents for generation of a tamoxifen-inducible cre (creERT2) are at hand and working (Fig 11). We have generated a floxExon1-PR construct and will shortly proceed with blastocyst injection (Fig 5). Through our collaborator Dr. Thomas Hamilton, we have identified a promoter (CK19) suitable for targeting OSE expression (specific to the epithelial compartment within the ovary) which will require additional specialized reagents (an inducible cre, and transplantation) for optimal tissue-specific studies and the analysis of OSE functions of PR in aging mice. We have assembled a very strong group of collaborators to tackle any issues arising during this project.

ABREVIATIONS:

Apoptosis	programmed cell death cause by double strand DNA breakage.
BrdU	a marker use to lable proliferating cells
cre	cre recombinase
creET2	a tamoxifen induced variant of cre recombinase
FITC	a fluoresent compound used to label antibodies
E2	Estrogen
ER	Estrogen Receptor.
HRT	hormone replacement therapy
LSC	Laser scanning microscope, a tool to count cells very accurately.
MOSE	mouse surfaxe ovarian epithelial cells
OC	oral contraceptive
OSE	ovarian surface epithelial cells
P	Progesterone
PR	Progesterone receptor
PRKO	mouse lineage that has a mutation in PR
R5020	a synthetic stimulator of PR signaling
RU486	an inhibitor of PR signaling
src	the src oncogene
tamoxifen	a inhibitor of ER at high concentrations. No effect on ER at concentrations that stimulate creET2
TUNEL	an assay to measure cell death by apoptosis

BIBLIOGRAPHY:

- Adams JY, M Johnson, M Sato, F Berger, SS Gambhir, M Carey, ML Iruela-Arispe, L Wu. 2002. Visualization of advanced human prostate cancer lesions in living mice by a targeted gene transfer vector and optical imaging. *Nat Med* 8: 891-7.
- Auersperg N, MI Edelson, SC Mok, SW Johnson, TC Hamilton. 1998. The biology of ovarian cancer. *Semin. Oncol.* 25:281-304.
- Auersperg N, AST Wong, K Choi, SK Kang, CK Leung. 2001. Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocr Rev* 22:255-288.
- Bao R, M Selvakumaran, TC Hamilton. 2002. Targeted gene therapy of ovarian cancer using an ovarian-specific promoter. *Gynecol Oncol* 84:228-34.
- Bast RCJ, CM Boyer, I Jacobs, FJ Xu, S Wu, J Wiener, M Kohler, A Berchuck. 1993. Cell growth regulation in epithelial ovarian cancer. *Cancer* 71:1597-601.
- Berchuck A. M Carney. 1997. Human ovarian cancer of the surface epithelium. *Biochem Pharmacol* 54:541-4.
- Bhaumik S, SS Gambhir. 2002. Optical imaging of Renilla luciferase reporter gene expression in living mice. *Proc Natl Acad Sci U S A* 99:377-82.
- Boonyaratanakornkit V, MP Scott, V Ribon, L Sherman, SM Anderson, JL Maller, WT Miller, DP Edwards. 2001. Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. *Mol Cell* 8:269-80.
- Brocard J, R Feil, P Chambon, D Metzger. 1998. A chimeric Cre recombinase inducible by synthetic, but not by natural ligands of the glucocorticoid receptor. *Nucleic Acids Res* 26:4086-90.
- Cancer and Steroid Hormone Study 1987. The reduction in risk of ovarian cancer associated with oral-contraceptive use. The Cancer and Steroid Hormone Study of the Centers for Disease Control and the National Institute of Child Health and Human Development. *N Engl J Med* 316:650-5.
- Cardiff RD, MR Anver, BA Gusterson, L Hennighausen, RA Jensen, MJ Merino, S Rehm, J Russo, FA Tavassoli, LM Wakefield, JM Ward, JE Green. 2000. The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. *Oncogene* 19:968-88.
- Casagrande JT, MT Pike, RK Ross, EW Louie, S Roy, BE Henderson. 1979. Incessant ovulation and ovarian cancer. *The Lancet* 2(8135):170-171.
- Christian J, H Thomas 2001. Ovarian cancer chemotherapy. *Cancer Treat Rev* 27:99-109.

- Conneely OM, JP Lydon. 2000. Progesterone receptors in reproduction: functional impact of the A and B isoforms. *Steroids* 65:571-7.
- Crosignani PG, G Testa, W. Vegetti, F Parazzini 1996. Ovarian activity during regular oral contraceptive use. *Contraception*. 54:271-3.
- Conneely OM, JP Lydon, F De Mayo, BW O'Malley. 2000. Reproductive functions of the progesterone receptor. *J Soc Gynecol Investig* 7:S25-32.
- Davies BR, N Auersperg, SD Worsley, BA Ponder. 1998. Transfection of rat ovarian surface epithelium with erb-B2/neu induces transformed phenotypes in vitro and the tumorigenic phenotype in vivo. *Am J Pathol* 152:297-306.
- Donehower LA 1996. The p53-deficient mouse: a model for basic and applied cancer studies. *Semin Cancer Biol.* 7:269-78.
- Elenbaas B, L Spirio, F Koerner, MD Fleming, D Zimonjic, J Donaher, NC Popescu, WC Hahn, RA Weinberg. 2001. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev* 15:50-65.
- Elson A, C Deng, J Campos-Torres, LA Donehower, P Leder. 1995. The MMTV/c-myc transgene and p53 null alleles collaborate to induce T-cell lymphomas, but not mammary carcinomas in transgenic mice. *Oncogene* 11:181-190.
- Fathalla MF 1971. Incessant ovulation: a factor in ovarian neoplasia? *The Lancet* 2:163.
- Feil R, J Wagner, D Metzger, P Chambon. 1997. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem Biophys Res Commun* 237:752-7.
- Giavazzi R, MI Nicoletti, RG Chirivi, I Hemingway, S Bernasconi, P Allavena, AJ Gearing. 1994. Soluble intercellular adhesion molecule-1 (ICAM-1) is released into the serum and ascites of human ovarian carcinoma patients and in nude mice bearing tumour xenografts. *Eur J Cancer* 30A:1865-1870.
- Godwin AK, JR Testa, LM Handel, Z Liu, LA Vanderveer, PA Tracey, TC Hamilton. 1992. Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in the etiology of ovarian cancer. *J Natl Cancer Inst* 84:592-601.
- Hamilton TC, RC Young, RF Ozols. 1984. Experimental model systems of ovarian cancer: applications to the design and evaluation of new treatment approaches. *Semin Oncol* 11:285-98.
- Hoskins WJ 1995. Prospectives on ovarian cancer: why prevent? *J Cell Biochem* 23:189-199.
- Hu Z, X Deng. 2000. [The effect of progesterone on proliferation and apoptosis in ovarian cancer cell]. *Zhonghua Fu Chan Ke Za Zhi* 35:423-6.

- Iyer M, L Wu, M Carey, Y Wang, A Smallwood, SS Gambhir. 2001. Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. *Proc Natl Acad Sci U S A* 98:14595-600.
- Jackson-Grusby L. 2002. Modeling cancer in mice. *Oncogene* 21:5504-14.
- Jensen JT, L Speroff. 2000. Health benefits of oral contraceptives. *Obstet Gynecol Clinics* 27:702-721.
- Jolles CJ, RS Freedman, LA Jones. 1983. Estrogen and progestogen therapy in advanced ovarian cancer: preliminary report. *Gynecol Oncol* 16:352-9.
- Kastan MB, Q Zhan, WS el-Deiry, F Carrier, T Jacks, WV Walsh, BS Plunkett, B Vogelstein, AJ Fornace, Jr. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* 71:587-97.
- Kaupilla A, P Vierikko, S Kivinen, F Stenback, R Vihko. 1983. Clinical significance of estrogen and progestin receptors in ovarian cancer. *Obstet Gynecol* 61:320-6.
- Kuhbandner S, S Brummer, D Metzger, P Chambon, F Hofmann, R Feil. 2000. Temporally controlled somatic mutagenesis in smooth muscle. *Genesis* 28:15-22.
- Lancaster JM, A Berchuk, ME Carney, JA Taylor. 1998. Progesterone receptor gene polymorphism and risk for breast and ovarian cancer. *Br J Cancer* 78:277.
- Lane TF, C Deng, A Elson, MS Lyu, CA Kozak, P Leder. 1995. Expression of Brca1 is associated with terminal differentiation of ectodermally and mesodermally derived tissues in mice. *Genes Dev* 9:2712-22.
- Lane TF, C Lin, MA Brown, E Solomon, P Leder. 2000. Gene replacement with the human BRCA1 locus: tissue specific expression and rescue of embryonic lethality in mice. *Oncogene* 19:4085-90.
- Lebel M, M Webster, WJ Muller, A Royal, J Gauthier AM Mes-Masson. 1995. Transgenic mice bearing the polyomavirus large T antigen directed by 2.1 kb of the keratin 19 promoter develop bronchiolar papillary tumors with progression to lung adenocarcinomas. *Cell Growth Differ* 6:1591-600.
- Liu CH, SH Chang, K Narko, OC Trifan, MT Wu, E Smith, C Haudenschild, TF Lane, T Hla. 2001. Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. *J Biol Chem* 276:18563-9.
- Liu Y, TS Ganesan. 2002. Tumour suppressor genes in sporadic epithelial ovarian cancer. *Reproduction* 123:341-53.
- Luongo C, WF Dove. 1996. Somatic genetic events linked to the Apc locus in intestinal adenomas of the Min mouse. *Genes Chromosomes Cancer* 17:194-198.

- Lydon JP, F DeMayo, O Conneely, BW O'Malley. 1996. Reproductive phenotypes of the progesterone receptor null mutant mouse. *J Steroid Biochem Mol Biol* 56:67-77.
- Lydon JP, FJ DeMayo, CR Funk, SK Mani, AR Hughes, CA Montgomery Jr, G Shyamala, OM Conneely, BW O'Malley. 1995. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev* 9:2266-78.
- Mao X, Y Fujiwara, SH Orkin. 1999. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. *Proc Natl Acad Sci U.S.A.* 96:5037-42.
- Matthews JC, K Hori, MJ Cormier. 1977. Substrate and substrate analogue binding properties of Renilla luciferase. *Biochemistry* 16:5217-20.
- McDonnell AC, WJ Murdoch. 2001. High-dose progesterone inhibition of urokinase secretion and invasive activity by SKOV-3 ovarian carcinoma cells: evidence for a receptor-independent nongenomic effect on the plasma membrane. *J Steroid Biochem Mol Biol* 78:185-91.
- McKenna NJ, DG Kieback, DN Carney, M Fanning, J McLinden, DR Headon. 1995. A germline TaqI restriction fragment length polymorphism in the progesterone receptor gene in ovarian carcinoma. *Br J Cancer* 71:451-5.
- Methot D, TL Reudelhuber, DW Silversides. 1995. Evaluation of tyrosinase minigene co-injection as a marker for genetic manipulations in transgenic mice. *Nucleic Acids Res* 23:4551-6.
- Munstedt K, J Steen, AG Knauf, T Buch, R von Georgi, FE Franke. 2000. Steroid hormone receptors and long term survival in invasive ovarian cancer. *Cancer* 89:1783-91.
- Murdoch WJ 1998a. Inhibition by oestradiol of oxidative stress-induced apoptosis in pig ovarian tissues. *J Reprod Fertil* 114:127-30.
- Murdoch WJ 1998b. Perturbation of sheep ovarian surface epithelial cells by ovulation: evidence for roles of progesterone and poly(ADP-ribose) polymerase in the restoration of DNA integrity. *J Endocrinol* 156:503-8.
- Murdoch WJ, RS Townsend, AC McDonnell. 2001. Ovulation-induced DNA damage in ovarian surface epithelial cells of ewes: prospective regulatory mechanisms of repair/survival and apoptosis. *Biol Reprod* 65:1417-24.
- Nagy A 2000. Cre recombinase: the universal reagent for genome tailoring. *Genesis* 26:99-109.
- Nagy A, L Mar. 2001. Creation and use of a Cre recombinase transgenic database. *Methods Mol Biol* 158:95-106.
- Narod SA, et al. 1998. Oral contraceptives and the risk of hereditary ovarian cancer. Hereditary Ovarian Cancer Clinical Study Group. *N Engl J Med* 339:424-8.

- Nash JD, R Ozols, J Smyth, TC Hamilton 1989. Estrogen and anti-estrogen effects on the growth of human epithelial ovarian cancer in vitro. *Obstet Gynecol* 73:1009-16.
- Nguyen, HN, HE Averette, W Hoskins, B Sevin, M Penalver, A Steren. 1993. Critical assessment of current international federation of gynecology and obstetrics staging system. *Cancer* 72:3007-3011.
- Orsulic S, Y Li, RA Soslow, LA Vitale-Cross, JS Gutkind, HE Varmus. 2002. Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. *Cancer Cell* 1:53-62.
- Overbeek PA, E Aguilar-Cordova, G Hanten, DL Schaffner, P Patel, RM Lebovitz, MW Lieberman. 1991. Coinjection strategy for visual identification of transgenic mice. *Transgenic Res* 1:31-7.
- Palmiter RD, RL Brinster. 1985. Transgenic mice. *Cell* 41:343-5.
- Phelps SM, J Lydon, B O'Malley, D Crews 1998. Regulation of male sexual behavior by progesterone receptor, sexual experience, and androgen. *Horm Behav* 34:294-302.
- Rajewsky K, H Gu, R Kuhn, UA Betz, W Muller, J Roes, F Schwenk. 1996. Conditional gene targeting. *J. Clin. Invest.* 98:600-603.
- Risch HA, NS Weiss, JL Lyon, JR Daling, JM Liff. 1983. Events of reproductive life and the incidence of epithelial ovarian cancer. *Am J Epidemiol* 117:128-39.
- Rodriguez GC, NP Nagarsheth, KL Lee, RC Bentley, DK Walmer, M Cline, RS Whitaker, P Isner, A Berchuck, RK Dodge, CL Hughes. 2002. Progestin-induced apoptosis in the Macaque ovarian epithelium: differential regulation of transforming growth factor-beta. *J Natl Cancer Inst* 94:50-60.
- Rodriguez-Manzaneque, JC, TF Lane, MA Ortega, RO Hynes, J Lawler, ML Iruela-Arispe. 2001. Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. *Proc Natl Acad Sci U S A* 98:12485-90.
- Rose GS, LM Tocco, GA Granger, PJ DiSaia, TC Hamilton, AD Santin, JC Hiserodt. 1996. Development and characterization of a clinically useful animal model of epithelial ovarian cancer in the Fischer 344 rat. *Am J Obstet Gynecol* 175:593-9.
- Rose GS, LM Tocco, GA Granger, JP DiSaia, TC Hamilton, AD Santin, JC Hiserodt, 1999. Response of human ovarian carcinoma cell lines to antiprogestin mifepristone *Oncogene*, 12:999-1003.
- Rosenberg L, JR Palmer, AG Zauber, ME Warshauer, JL Lewis, BL Strom, S Harlap, S Shapiro. 1994. A case-control study of oral contraceptive use and invasive ovarian cancer. *Am J Epidemiol* 1994;139:654-61. 139.

- Rosling MA, JR Daling, NS Weiss, DE Moore, SG Self. 1994. Ovarian tumors in a cohort of infertile women. *New Engl J Med* 331:771-776.
- Rowe SM, SJ Coughlan, NJ McKenna, E Garrett, DG Kieback, DN Carney, DR Headon. 1995. Ovarian carcinoma-associated TaqI restriction fragment length polymorphism in intron G of the progesterone receptor gene is due to an Alu sequence insertion. *Cancer Res* 55:2743-5.
- Runnebaum IB, S Wang-Gohrke, D Vesprini, R Kreienberg, H Lynch, R Moslehi, P Ghadirian, B Weber, AK Godwin, H Risch, J Garber, C Lerman, OI Olopade, WD Foulkes, B Karlan, E Warner, B Rosen, T Rebbeck, P Tonin, MP Dube, DG Kieback, SA Narod. 2001. Progesterone receptor variant increases ovarian cancer risk in BRCA1 and BRCA2 mutation carriers who were never exposed to oral contraceptives. *Pharmacogenetics* 11:635-8.
- Selvakumaran M, R Bao, AP Crijns, DC Connolly, JK Weinstein, TC Hamilton. 2001. Ovarian epithelial cell lineage-specific gene expression using the promoter of a retrovirus-like element. *Cancer Res* 61:1291-5.
- Takaku K, M Oshima, H Miyoshi, M Matsui, MF Seldin, MM Taketo. 1998. Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell* 92:645-56.
- Tanyi JL, R. Lapushin, A Eder, N Auersperg, FH Tabassam, JA Roth, J Gu, B Fang, GB Mills, J Wolf. 2002. Identification of tissue- and cancer-selective promoters for the introduction of genes into human ovarian cancer cells. *Gynecol Oncol* 85:451-8.
- Thyagarajan B, MJ Guimaraes, AC Groth, MP Calos. 2000. Mammalian genomes contain active recombinase recognition sites. *Gene* 244:47-54.
- Tong D, G Fabjani, G Heinze, A Obermair, S Leodolter, R Zeillinger. 2001. Analysis of the human progesterone receptor gene polymorphism progins in Austrian ovarian carcinoma patients. *Int J Cancer* 95:394-7.
- Tortolero-Luna G, MF Mitchell. 1995. The epidemiology of ovarian cancer. *J Cell Biochem* 23(Suppl):200-207.
- Sauer B. 1998. Inducible gene targeting in mice using the Cre/lox system. *Methods*. 14:381-392.
- Venn A, L Watson, F Bruinsma, G Giles, DL Healy. 1999. Risk of cancer after use of fertility drugs with in-vitro fertilisation. *Lancet* 354:1586-1590.
- Vooijs M, J Jonkers, S Lyons, A Berns 2002. Noninvasive imaging of spontaneous retinoblastoma pathway-dependent tumors in mice. *Cancer Res* 62:1862-7.

- Wang-Gohrke S, J Chang-Claude, H Becher, DG Kieback, IB Runnebaum. 2000. Progesterone receptor gene polymorphism is associated with decreased risk for breast cancer by age 50. *Cancer Res* 60:2348-50.
- Weiss NS, LS Cook, DC Farrow, KA Rosenblatt. 1996. Ovarian cancer. In *Cancer epidemiology and prevention* (ed. D. Schottenfeld and J.F.J. Fraumeni), pp. 1040-57. Oxford University Press, New York (NY).
- Weiss NS, JL Lyon, JM Liff, WM Vollmer, JR Daling. 1981. Incidence of ovarian cancer in relation to the use of oral contraceptives. *Int J Cancer* 28:669-71.
- Westphal CH, KP Hoyes, CE Canman, X Huang, MB Kastan, JH Hendry, P Leder. 1998. Loss of atm radiosensitizes multiple p53 null tissues. *Cancer Res* 58:5637-9.
- Whittemore AS, R Harris, J Intyre. 1992. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. II. Invasive epithelial ovarian cancers in white women. *Am J Epidemiol* 136:1184-1203.
- Whittemore AS, V McGuire. 2002. Analysis of the human progesterone receptor gene polymorphism PROGENS in Austrian ovarian cancer patients. *Int J Cancer* 101:202; discussion 203.
- Wright JW, S Toth-Fejel, RL Stouffer, KD Rodland. 2002. Proliferation of rhesus ovarian surface epithelial cells in culture: lack of mitogenic response to steroid or gonadotropic hormones. *PG - 2198-207. Endocrinology* 143.
- Wunderlich FT, H Wildner K Rajewsky, F Edenhofer. 2001. New variants of inducible Cre recombinase: a novel mutant of Cre-PR fusion protein exhibits enhanced sensitivity and an expanded range of inducibility. *Nucleic Acids Res* 29:E47.
- Zhang L, J Adams, E Billick, R Ilagan, M Iyer, K Le, A Smallwood, SS Gambhir, M Carey, L Wu. 2002. Molecular engineering of a two-step transcription amplification (TSTA) system for transgene delivery in prostate cancer. *Mol Ther* 5:223-32.